

Neurobiology

# Microglia Activated with the Toll-Like Receptor 9 Ligand CpG Attenuate Oligomeric Amyloid $\beta$ Neurotoxicity in *in Vitro* and *in Vivo* Models of Alzheimer's Disease

Yukiko Doi,\* Tetsuya Mizuno,\* Yuki Maki,\*  
Shijie Jin,\* Hiroyuki Mizoguchi,<sup>†</sup>  
Masayoshi Ikeyama,<sup>‡</sup> Minoru Doi,<sup>‡</sup>  
Makoto Michikawa,<sup>§</sup> Hideyuki Takeuchi,\*  
and Akio Suzumura\*

From the Department of Neuroimmunology\* and the Futuristic Environmental Simulation Center,<sup>†</sup> Research Institute of Environmental Medicine, Nagoya University, Nagoya; the Department of Materials Science and Engineering,<sup>‡</sup> Nagoya Institute of Technology, Nagoya; and the Department of Alzheimer's Disease Research,<sup>§</sup> National Institute for Longevity Sciences, National Center for Geriatrics and Gerontology, Aichi, Japan

**Soluble oligomeric amyloid  $\beta$  (oA $\beta$ ) 1-42 causes synaptic dysfunction and neuronal injury in Alzheimer's disease (AD). Although accumulation of microglia around senile plaques is a hallmark of AD pathology, the role of microglia in oA $\beta$ 1-42 neurotoxicity is not fully understood. Here, we showed that oA $\beta$  but not fibrillar A $\beta$  was neurotoxic, and microglia activated with unmethylated DNA CpG motif (CpG), a ligand for Toll-like receptor 9, attenuated oA $\beta$ 1-42 neurotoxicity in primary neuron-microglia co-cultures. CpG enhanced microglial clearance of oA $\beta$ 1-42 and induced higher levels of the antioxidant enzyme heme oxygenase-1 in microglia without producing neurotoxic molecules such as nitric oxide and glutamate. Among subclasses of CpGs, class B and class C activated microglia to promote neuroprotection. Moreover, intracerebroventricular administration of CpG ameliorated both the cognitive impairments induced by oA $\beta$ 1-42 and the impairment of associative learning in Tg2576 mouse model of AD. We propose that CpG may be an effective therapeutic strategy for limiting oA $\beta$ 1-42 neurotoxicity in AD. (*Am J Pathol* 2009, 175:2121-2132; DOI: 10.2353/ajpath.2009.090418)**

The senile plaque is a pathological hallmark of Alzheimer's disease (AD). Fibrillar amyloid  $\beta$  (fA $\beta$ ), a major component of senile plaques, induces tau hyperphosphorylation and neuronal dystrophy.<sup>1,2</sup> Soluble oligomeric A $\beta$  (oA $\beta$ ) has been reported to exhibit higher neurotoxicity than fA $\beta$ . Naturally secreted oA $\beta$  inhibits hippocampal long-term potentiation and disrupts synaptic plasticity in rats *in vivo*.<sup>3</sup> In addition, oA $\beta$  induces neuronal reactive oxygen species (ROS) through a mechanism requiring *N*-methyl-D-aspartate receptor activation.<sup>4</sup> Exposure to oA $\beta$  induces rapid and massive neuronal death, while fA $\beta$  is required at higher concentrations and for longer incubations to cause neuronal dystrophy.<sup>5</sup>

Microglia, macrophage-like cells in the central nervous system, cluster both in and around senile plaques and have been proposed to have pivotal roles in the pathogenesis of AD. Microglia activated with A $\beta$  may be involved in the inflammatory component of AD.<sup>6</sup> Both fA $\beta$  and oA $\beta$  stimulate microglial secretion of proinflammatory cytokines, chemokines, complement components, and free radicals.<sup>7</sup> However, microglia also perform neu-

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Y.D. and T.M. contributed equally to this paper.

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Address reprint requests to Tetsuya Mizuno, Department of Neuroimmunology, Research Institute of Environmental Medicine, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan. E-mail: tmizuno@riem.nagoya-u.ac.jp.

roprotective functions such as releasing neurotrophic factors<sup>8</sup> and phagocytosing and degrading A $\beta$ .<sup>9,10</sup>

Toll-like receptor (TLR) ligands enhance microglial phagocytosis of A $\beta$ . Peptidoglycan, a TLR2 ligand, and unmethylated DNA CpG motifs (CpG), a TLR9 ligand, increase A $\beta$  phagocytosis through the G protein-coupled formyl peptide receptor-like 2.<sup>11,12</sup> Similarly, the TLR4 ligand lipopolysaccharide increases phagocytosis through the CD14 receptor.<sup>13</sup> However, microglia activated with TLR ligands also produce neurotoxic molecules such as proinflammatory cytokines, nitric oxide (NO), ROS, and peroxynitrite.<sup>14</sup> In particular, lipopolysaccharide-activated microglia produce a large amount of glutamate, a neurotransmitter but also potent neurotoxin.<sup>15</sup> Thus, factors that increase microglial clearance of oA $\beta$  without producing inflammatory mediators are candidates for the treatment of AD.

Here, we investigated the role of microglia in neurotoxicity mediated by oA $\beta$ 1-42. We found that microglia activated with a low dose of CpG attenuated the neurotoxic effects of oA $\beta$ 1-42 without producing other neurotoxic molecules *in vitro*. Moreover, intracerebroventricular (ICV) administration of CpG ameliorated the cognitive impairment induced by ICV injection of oA $\beta$ 1-42 and the impairment of associative learning in Tg2576 mouse model of AD.

## Materials and Methods

### Cell Culture

The protocols for animal experiments were approved by the Animal Experiment Committee of Nagoya University. Primary neuronal cultures were prepared from the cortices of embryonic day 17 (E17) C57BL/6 mice embryos as described previously.<sup>8</sup> Briefly, cortical fragments were dissociated into single cells in dissociation solution (Sumitomo Bakelite, Akita, Japan) and resuspended in Nerve Culture Medium (Sumitomo Bakelite). Neurons were plated onto 12-mm-polyethyleneimine-coated glass coverslips (Asahi Techno Glass, Chiba, Japan) at a density of  $5 \times 10^4$  cells/well in 24-well multidishes and incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. The purity of the cultures was >95% as determined by NeuN-specific immunostaining. Microglia were isolated on 14 days *in vitro* with the "shaking off" method previously described from primary mixed glial cell cultures prepared from newborn C57BL/6 mice.<sup>16</sup> Cultures were 97 to 100% pure as determined by Fc receptor-specific immunostaining and were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, 5  $\mu$ g/ml bovine insulin, and 0.2% glucose. Microglia were plated at a density of  $7 \times 10^4$  cells/well in 8-well glass slides or at a density of  $7 \times 10^4$  cells/well in 48-well multidishes. Neuron-microglia co-cultures were prepared as follows:  $1 \times 10^5$  microglia in 100  $\mu$ l of neuronal medium were added to neuronal cultures ( $5 \times 10^4$  neuronal cells) on 13 day *in vitro* in 24-well multidishes.

### Preparations of A $\beta$ Solutions

fA $\beta$ 1-42 was prepared as described previously.<sup>17</sup> Briefly, synthetic human A $\beta$ 1-42 (Peptide Institute, Osaka, Japan) was dissolved in 0.02% ammonia solution at a concentration of 250  $\mu$ mol/L, diluted to 25  $\mu$ mol/L in PBS, and incubated at 37°C for 24 hours. oA $\beta$ 1-42 was prepared as described previously.<sup>18</sup> Briefly, A $\beta$ 1-42 was dissolved to 1 mmol/L in 100% 1,1,1,3,3,3-hexafluoro-2-propanol. 1,1,1,3,3,3-Hexafluoro-2-propanol was dried by the vacuum desiccator and resuspended to 5 mmol/L in DMSO. To form oligomers, amyloid peptide was diluted to a final concentration of 100  $\mu$ mol/L with Ham's F-12 and incubated at 4°C for 24 hours and then immediately added to cultures at a final concentration 5  $\mu$ mol/L.

### Transmission Electron Microscopy

To assess quaternary structures of A $\beta$ , oA $\beta$ 1-42 and fA $\beta$ 1-42 solutions were spread on carbon-coated grids. Negative staining was performed with 3% phosphotungstic acid (pH 7.0). Proteins were then examined with a JEM-2000ExII Electron Microscope with an acceleration voltage of 160 kV.

### Thioflavin T Assay

Optimum fluorescence measurements of amyloid fibrils were obtained at excitation and emission wavelength of 446 and 490 nm, respectively, with a reaction mixture containing 5  $\mu$ mol/L thioflavin T (Nakalai tesque, Kyoto, Japan) and 50 mmol/L glycine-NaOH buffer (pH 8.5). Ten microliters of oA $\beta$ 1-42 or fA $\beta$ 1-42 solution was mixed with 100  $\mu$ l of the reaction mixture, respectively.

### Measurement of Heme Oxygenase-1, Interleukin-10, Matrix Metalloproteinase-9, Tumor Necrosis Factor- $\alpha$ , NO, and Glutamate

To measure factors produced by microglia treated with CpG and oA $\beta$ 1-42, microglia were plated at a density of  $7 \times 10^4$  cells/well (300  $\mu$ l) in 48-well multidishes and then treated with 100 nmol/L CpG-DNA (HyCult Biotechnology, Uden, Netherlands), 100 nmol/L class A CpG (synthetic oligodeoxynucleotides (ODNs) 1585), class B CpG (ODN 1668), and class C CpG (ODN 2395). These CpG subtypes were from Alexis Biochemicals (San Diego, CA). After 3 hours of treatment with CpG, 5  $\mu$ mol/L oA $\beta$ 1-42 was added for 24 hours. Supernatants from microglia were assessed by ELISA kits for tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin (IL)-10 (BD Pharmingen, Franklin Lakes, NJ) and matrix metalloproteinase (MMP)-9 (R&D Systems, Minneapolis, MN). Cell extracts from microglia in extraction buffer (1% Nonidet P-40 in PBS) were measured for heme oxygenase-1 (HO-1) with an ELISA kit (Takara Bio, Mie, Japan). Measurement of NO was determined using the Griess reaction.<sup>19</sup> To measure glutamate, Glutamate Assay Kit colorimetric assay (Yamasa, Tokyo, Japan) was used as described previously.<sup>20</sup>

The mRNA expression of HO-1 was assessed by RT-PCR. Briefly, total RNA was extracted using the guanidinium thiocyanate method (RNeasy Mini Kit; Qiagen, Valencia, CA). cDNAs were generated by RT-PCR using SuperScript II (Invitrogen, Carlsbad, CA) and Ampli TaqDNA polymerase (Applied Biosystems, Branchburg, NJ) in the presence of the specific primers. HO-1 sense, 5'-CTATGTAAAGCGTCTCCA-3'; and HO-1 antisense, 5'-GTCTTTGTGTTCTCTGTC-3'.

### Measurement of ROS

To measure ROS in neuron-microglia co-cultures, we used the acetate ester form of 2',7'-dichlorofluorescein diacetate (H<sub>2</sub>DCFDA-AM) probe (Invitrogen). After neuron-microglia co-cultures were treated with or without 100 nmol/L CpG for 3 hours, cells were loaded with dye by replacing media with fresh nerve culture medium containing 5  $\mu$ mol/L H<sub>2</sub>DCFDA-AM for 30 minutes. After washing, culture medium containing 5  $\mu$ mol/L oA $\beta$ 1-42 was added and the fluorescence of the wells was measured. Fluorescent measurements were made using a Wallac 1420 ARVOMX (PerkinElmer Japan, Yokohama, Japan).

### Immunocytochemistry

Neuronal, microglial, and neuron-microglia co-cultures were fixed with 4% paraformaldehyde for 30 minutes at room temperature, then blocked with 5% normal goat serum in PBS and permeabilized with 0.3% Triton X-100. Neurons were stained with rabbit polyclonal anti-microtubule-associated protein (MAP)-2 antibody (1/500; Chemicon, Temecula, CA) and secondary antibodies conjugated to Alexa 488 (1/1000; Invitrogen). Synthetic A $\beta$  was stained with a mouse monoclonal anti-A $\beta$  antibody (4G8) (1/1000; Chemicon) and secondary antibodies conjugated to Alexa 568 or 647. Microglia were stained with phycoerythrin-conjugated rat anti-mouse CD11b monoclonal antibody (1/300; BD Pharmingen) before fixation. Images were analyzed with a deconvolution fluorescent microscope system (BZ-8000; Keyence, Osaka, Japan). To assess neuronal death induced by A $\beta$ , purified neurons (5  $\times$  10<sup>4</sup> cells/well) were plated in 24-well multidishes. A total of 5  $\mu$ M oA $\beta$ 1-42 or fA $\beta$ 1-42 was added to the cultures on 13 days *in vitro* for 24 hours. To assess neuronal death in neuron-microglia co-cultures, 3 hours after treatment with or without TLR ligands, 5  $\mu$ mol/L oA $\beta$ 1-42 was added to cultures for 24 hours. Surviving neurons were identified by cytoskeletal morphology of neurons as described previously.<sup>8</sup> Viable neurons stained strongly with an anti-MAP-2 antibody, whereas damaged neurons stained more weakly. The number of MAP-2-positive neurons was counted in representative areas per well. More than 200 neurons were examined in each of five independent trials by a scorer blind to the experimental condition. The number of untreated viable neurons was normalized to 100%.

### Western Blotting

Neuronal cultures were treated with 5  $\mu$ mol/L oA $\beta$ 1-42 for 24 hours. Neuron-microglia co-cultures were pre-treated with CpG for 3 hours before addition of 5  $\mu$ mol/L oA $\beta$ 1-42 for 24 hours. The supernatants of these cultures were collected. oA $\beta$  in 10-month-old-Tg 2576 mouse brain was extracted from the soluble, extracellular-enriched fraction as described previously.<sup>21</sup> Hemi-forebrains were harvested in 500  $\mu$ l of solution containing 50 mmol/L Tris-HCl (pH 7.6), 0.01% Nonidet P-40, 150 mmol/L NaCl, 2 mmol/L EDTA, 0.1% SDS, and protease inhibitor mixture (Sigma-Aldrich, St. Louis, MO). Soluble, extracellular-enriched proteins were collected from mechanically homogenized lysates following centrifugation for 5 minutes at 3000 rpm.

Collected samples were mixed with sample buffer (200 mmol/L Tris-HCl, 8% SDS, and 1% glycerol). Proteins were separated on a 5 to 20% Tris-glycine SDS-polyacrylamide gel and transferred to Hybond-P polyvinylidene difluoride membrane (GE Healthcare UK, Buckinghamshire, UK). Membranes were blocked with 1% skim milk in Tris-buffered saline (TBS) containing 0.05% Tween20 (TBS-T). Blots were incubated in mouse anti-A $\beta$  monoclonal antibody (6E10) (1/1000; Chemicon) diluted in 1% skim milk overnight at 4°C. Subsequently, membranes were washed in TBS-T 3  $\times$  5 minutes and incubated with a horseradish peroxidase-conjugated anti-mouse IgG (1/5000; GE Healthcare) diluted in 1% skim milk for 1 hour. After washing in TBS-T for 1  $\times$  15 minutes, 2  $\times$  5 minutes, and TBS for 1  $\times$  5 minutes, signals were visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL). The intensity of the bands was calculated by using CS Analyzer 1.0 (Atto, Tokyo, Japan).

### Novel-Object Recognition Test in oA $\beta$ 1-42-Induced Cognitive Impairment Mouse Model

oA $\beta$  (300 pmol/3  $\mu$ l), CpG (100 nmol/L), or both oA $\beta$  and CpG were ICV injected as described previously.<sup>22,23</sup> The vehicle (PBS) was injected as the control. Briefly, a microsyringe with a 28-gauge stainless-steel needle 3.0 mm long was used for these experiments. C57/BL6 mice were anesthetized lightly with ether, and the needle was inserted unilaterally 1 mm to the right of the midline point equidistant from each eye, at an equal distance between the eyes and the ears and perpendicular to the plane of the skull. A single injection of 3  $\mu$ l of peptide or vehicle was delivered gradually over 3 minutes. The injection site was confirmed in preliminary experiments. Neither insertion of the needle nor the volume of injection had a significant influence on survival and behavioral responses or cognitive functions.

The novel-object recognition test (NORT) was performed 7 to 8 days after ICV injection of oA $\beta$ 1-42 or CpG as described previously.<sup>24,25</sup> The experimental apparatus consisted of a plexiglas open-field box (30  $\times$  30  $\times$  35 high cm), with a sawdust-covered floor. The apparatus

was located in a sound-attenuated room and was illuminated with a 60 lux light source. The NORT procedure consisted of three sessions: habituation, training, and retention. Each mouse was individually habituated to the box with 10 minutes of exploration in the absence of objects for 3 consecutive days (habituation session, days 4 to 6). During the training session, two novel objects were symmetrically fixed to the floor of the box, 8 cm from the walls, and each animal was allowed to explore in the box for 10 minutes (day 7). The objects were constructed from a golf ball, wooden column, and wooden triangular pyramid. They were different in shape and color but similar in size. An animal was considered to be exploring the object when its head was facing the object or it was touching or sniffing the object. The time spent exploring each object was recorded. After training, mice were immediately returned to their home cages. During the retention sessions (day 8), the animals were placed back into the same box 24 hours after the training session, but one of the familiar objects used during training had been replaced with a novel object. The animals were then allowed to explore freely for 5 minutes, and the time spent exploring each object was recorded. Throughout the experiments, the objects were used in a counterbalanced manner. A preference index in the retention session, a ratio of the amount of time spent exploring the novel object over the total time spent exploring both objects, was used to measure cognitive function. In the training session, the preference index was calculated as a ratio of the time spent exploring the object that was replaced by the novel object in the retention session over the total time exploring.

### *Cued and Contextual Fear-Conditioning Tests in Tg 2576 Mouse Model of AD*

Cued and contextual fear conditioning tests were performed at 10 months of age according to previous report,<sup>26</sup> with minor modifications. For measuring basal levels of freezing response (preconditioning phase), mice were individually placed in a neutral cage (a block plexiglas box with abundant wood chips, 30 × 30 × 40 high cm) for 1 minute, then in the conditioning cage (a transparent plexiglas box, 30 × 30 × 40 high cm) for 2 minutes. For training (conditioning phase), mice were placed in the conditioning cage, then a 15-second tone (80 dB) was delivered as a conditioned stimulus. During the last 5 seconds of the tone stimulus, a foot shock of 0.6 mA was delivered as an unconditioned stimulus through a shock generator (Neuroscience Idea). This procedure was repeated four times with 15-second intervals. Cued and contextual tests were performed 1 day after fear conditioning. For the contextual test, mice were placed in the conditioning cage, and the freezing response was measured for 2 minutes in the absence of the conditioned stimulus. For the cued test, the freezing response was measured in the neutral cage for 1 minute in the presence of a continuous-tone stimulus identical to the conditioned stimulus.

Stereotaxic injection was used for these experiments. Mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) before stereotaxic implantation of a microinjection cannula into the right lateral ventricle (anteroposterior, -0.3 mm, and mediolateral, +1.0 mm, from the bregma, and dorsoventral, +2.5, from the skull according to the atlas of Franklin and Paxinos).<sup>27</sup> CpG was dissolved in PBS at a concentration of 10 or 100 nmol/L and was injected at a volume of 3  $\mu$ l for 3 minutes. Same volume of PBS was injected to vehicle mouse. A week after injection, behavioral experiment was performed.

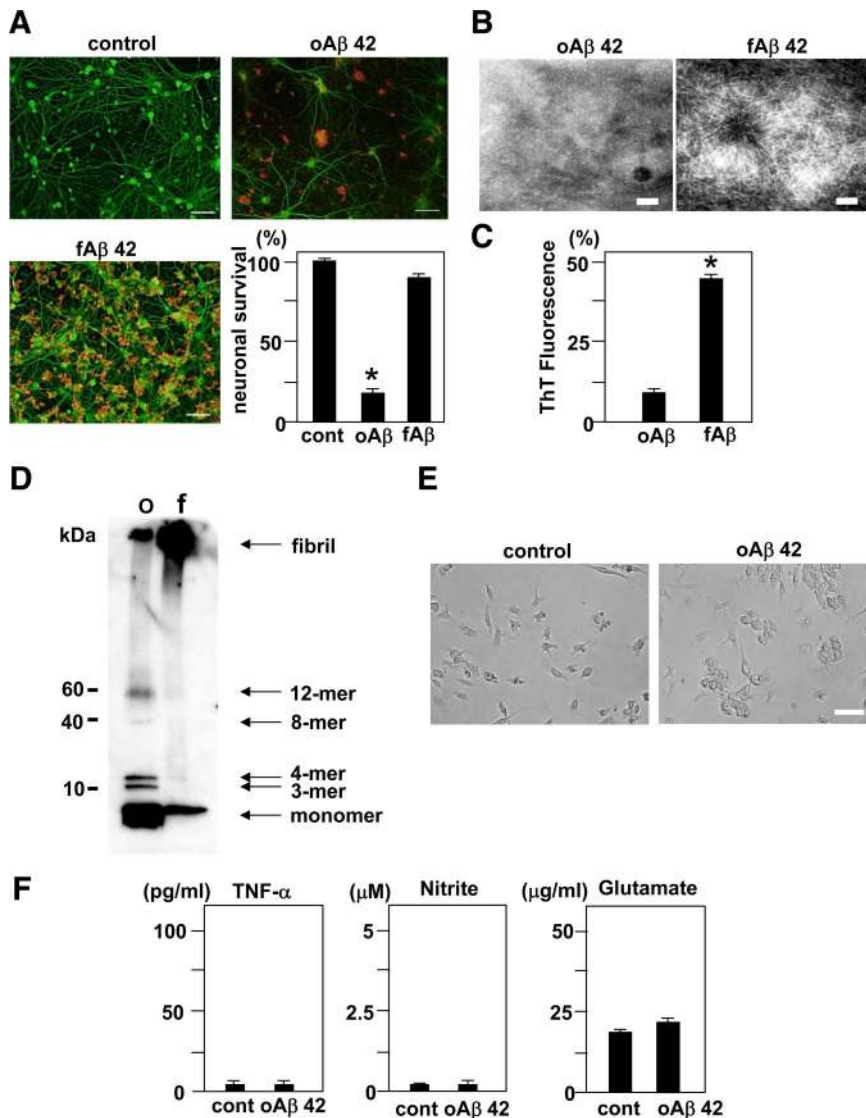
### *Immunohistochemistry*

Immunohistochemistry was performed on brain tissue of mice after cued and contextual fear-conditioning test. Mice were transcardially perfused with ice-cold borate-buffered 4% paraformaldehyde under deep anesthesia. The brains were rapidly removed after decapitation. Brains were then postfixed overnight in periodate lysine paraformaldehyde, equilibrated in phosphate buffered 20% sucrose for 48 hours, and embedded into Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan) and frozen at -80°C overnight. Coronal brain sections (20  $\mu$ m) were cut with a cryostat. The sections were permeabilized with 1% Triton X-100 after blocking with 10% normal goat serum for 30 minutes. The cell nucleus was stained with Hoechst 33342 (1  $\mu$ g/ml; Invitrogen). A $\beta$  was stained with mouse monoclonal anti-A $\beta$  antibody (4G8) (1/1000; Chemicon) and secondary antibodies conjugated to Alexa 488. Microglia were stained with a rat anti-mouse CD11b monoclonal antibody (1/1000; AbD Serotec, Oxford, UK) and secondary antibodies conjugated to Alexa 568. Images were collected and analyzed with a deconvolution fluorescent microscope system. A $\beta$  load in immunostained tissue sections were quantified using BZ-analyzer (Keyence) as reported previously.<sup>28</sup> Seven sections were analyzed per animal. Total A $\beta$  burden was quantified for the cortex and for the hippocampus on coronal plane sections stained with the monoclonal antibody 4G8. The cortical area was dorsomedial from the cingulate cortex and extended ventrolaterally to the rhinal fissure within the right hemisphere. Test areas (640  $\mu$ m × 480  $\mu$ m) were randomly selected. Total A $\beta$  burden was calculated as the percentage of test area occupied by A $\beta$ . Hippocampal measurements (600 × 600  $\mu$ m) were performed similarly to the cortical analysis.

### *Statistical Analysis*

Statistical significance of the biochemical experiments and the behavioral data were assessed with one-way analysis of variance, followed by post hoc Tukey test or Newman-Keuls test using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA).





**Figure 1.** Neurotoxicity and morphologies of oA $\beta$ 1-42 and fA $\beta$ 1-42, and microglial response to oA $\beta$ 1-42. **A:** The evaluation of neurotoxicity induced by oA $\beta$ 1-42 and fA $\beta$ 1-42. Neuronal cultures were treated with 5  $\mu$ mol/L oA $\beta$ 1-42 or fA $\beta$ 1-42 for 24 hours. Neurons were stained with an anti-MAP-2 antibody (green). A $\beta$  was stained with a mouse anti-amyloid  $\beta$  protein monoclonal antibody (4G8) (red). oA $\beta$ 1-42 exhibited more striking neurotoxicity than fA $\beta$ 1-42. Scale bar, 50  $\mu$ m. Neuronal survival rate in oA $\beta$ 1-42 treatment significantly decreased. \* $P$  < 0.05 as compared with the rate in control cultures. Each column indicates the mean  $\pm$  SEM ( $n$  = 5). **B:** Images of oA $\beta$ 1-42 (left) and fA $\beta$ 1-42 (right) collected with an electron microscope. Scale bar, 100 nm. **C:** Thioflavin T assay for oA $\beta$ 1-42 and fA $\beta$ 1-42. \* $P$  < 0.05 as compared with the value of oA $\beta$ 1-42. **D:** Western blot analysis of oA $\beta$ 1-42 and fA $\beta$ 1-42. oA $\beta$ 1-42 (o) contained monomers, small oligomeric trimers (3-mer) and tetramers (4-mer), and the larger oligomers (octamers (8-mer) and dodecamers (12-mer)), whereas fA $\beta$ 1-42 (f) contained monomers and fibrils. **E:** Microglial cultures were treated with or without 5  $\mu$ mol/L oA $\beta$ 1-42 for 24 hours. In a phase contrast, oA $\beta$ 1-42 induced microglial adhesion. Scale bar, 50  $\mu$ m. **F:** The measurement of TNF- $\alpha$  (left), nitrite (middle), and glutamate (right) produced by microglia activated with oA $\beta$ 1-42. Microglial cultures were treated with 5  $\mu$ mol/L oA $\beta$ 1-42 for 24 hours. Each column indicates the mean  $\pm$  SEM ( $n$  = 7).

## Results

### Neurotoxicity of oA $\beta$ 1-42

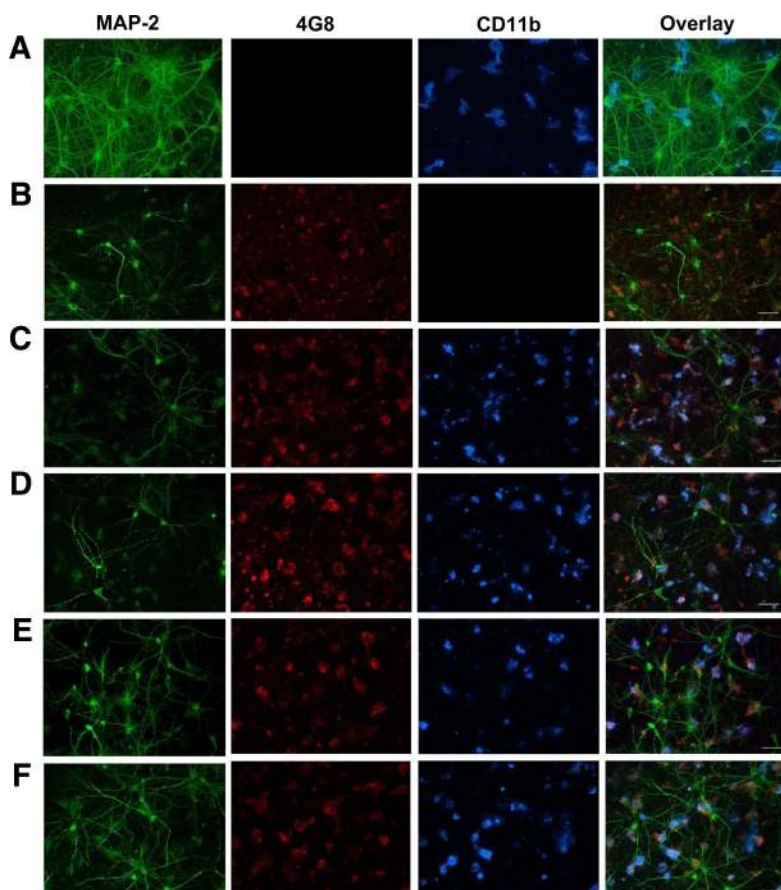
First, we investigated the toxic effects of oA $\beta$  and fA $\beta$  on primary cortical neurons. Administration of 5  $\mu$ mol/L oA $\beta$ 1-42 to cortical cultures on DIV 13 for 24 hours resulted in significant neuronal death. The network of MAP-2-positive dendrites collapsed and neuronal survival decreased to 20% (Figure 1A). In contrast, administration of fA $\beta$ 1-42 did not induce neuronal cell death, although A $\beta$  deposition was observed on dendrites (Figure 1A). Thus, oA $\beta$ 1-42 exhibits a more potent neurotoxicity than fA $\beta$ 1-42. Both oA $\beta$ 1-40 and fA $\beta$ 1-40 did not induce neuronal cell death (Supplemental Figure S1, see <http://ajp.amjpathol.org>). We evaluated the morphology of oA $\beta$ 1-42 and fA $\beta$ 1-42 by transmission electron microscopy. We observed fine spherical particles of oA $\beta$ 1-42 and fibril formation by fA $\beta$ 1-42 (Figure 1B). The fluorescence of Thioflavin T, a marker for amyloid fibril formation, was associated with fA $\beta$ 1-42 (Figure 1C). Western blotting

with an antibody directed against A $\beta$  (6E10) revealed that a solution of oA $\beta$ 1-42 contained monomers, trimers (3-mer), tetramers (4-mer), and larger oligomers (octamers (8-mer) and dodecamers (12-mer)). In contrast, a solution of fA $\beta$ 1-42 contained monomers and fibrils, but not oligomers (Figure 1D).

In primary microglial culture, administration of 5  $\mu$ mol/L oA $\beta$ 1-42 for 24 hours induced microglial adhesion (Figure 1E), but did not induce the production of neurotoxic mediators such as TNF- $\alpha$ , glutamate, or nitrite, a stable breakdown product of NO (Figure 1F).

### Microglia Activated with CpG Attenuate the Neurotoxicity Induced by oA $\beta$ 1-42

To define the role of microglia in the neurotoxicity of oA $\beta$ 1-42, we evaluated neuronal survival in neuron-microglia co-cultures. Neurons stained with anti-MAP-2 antibody exhibited no detectable morphological abnormalities and possessed intact cell bodies and dendrites, and



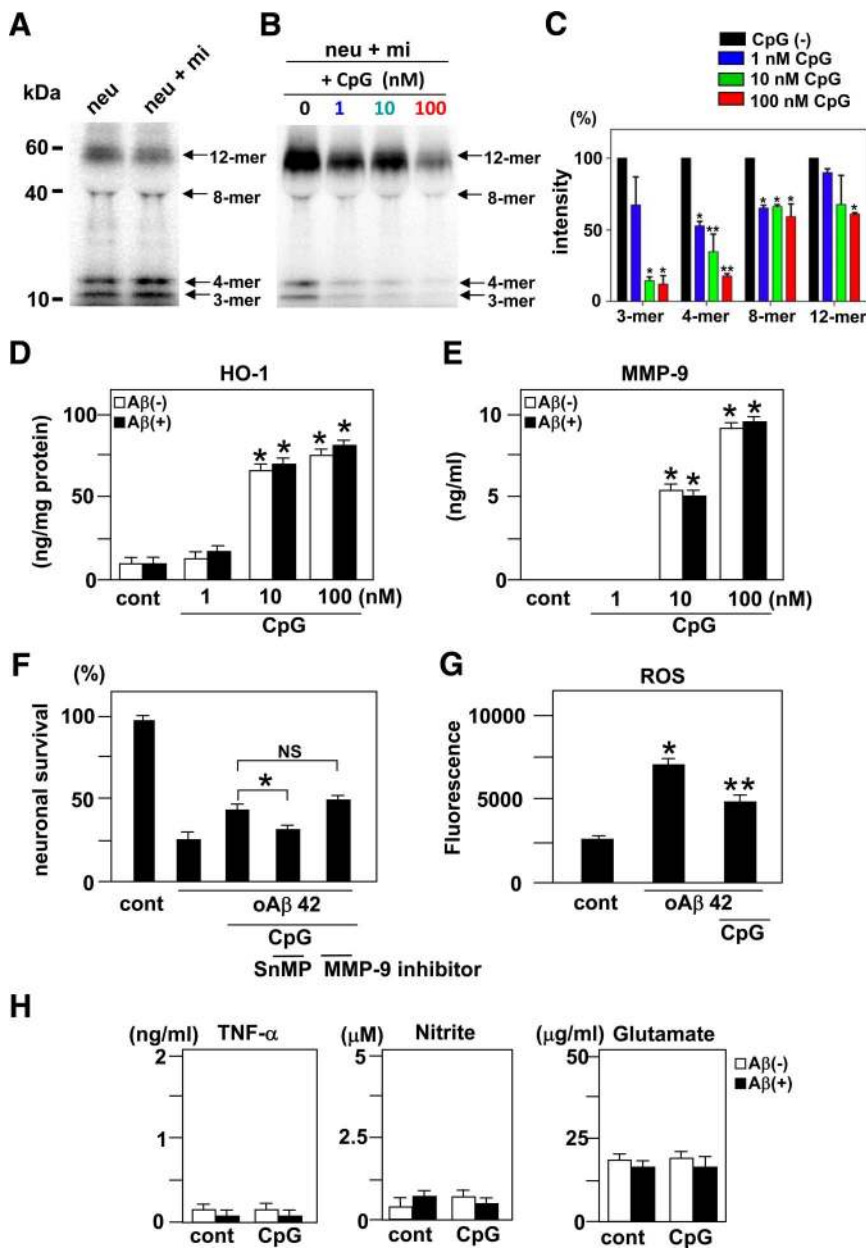
**Figure 2.** Protective effect of microglia activated with CpG against oAβ1-42 neurotoxicity. **A:** Representative deconvolution fluorescent images of control neuron-microglia co-cultures (1:2 of neuron: microglia). **B:** Neuronal cultures treated with 5 μmol/L oAβ1-42. **C:** Neuron-microglia co-cultures (1:2 neuron to microglia) treated with oAβ1-42. **D:** Neuron-microglia co-cultures stimulated with 1 nmol/L CpG and oAβ1-42 (**E**) or with 10 nmol/L CpG and oAβ1-42 (**F**) or with 100 nmol/L CpG and oAβ1-42. After 3 hours of treatment with CpG, cultures were treated with oAβ1-42 for 24 hours. Neurons were stained with an anti-MAP-2 antibody (green). Aβ was stained with 4G8 (red) and microglia were stained with a phycoerythrin-conjugated anti-CD11b antibody (blue). Scale bar, 50 μm. **G:** Neuronal survival rate was quantified as the percentage of intact neurons following treatment relative to control wells. The viability of untreated neurons (control) was normalized to 100%. \**P* < 0.05 as compared with the survival rate of neuron-microglia co-cultures treated with oAβ1-42. Each column indicates the mean ± SEM (*n* = 7).

microglia stained with anti-CD11b antibody were also intact in unstimulated co-cultures (Figure 2A). After treatment of neuronal cultures with 5 μmol/L oAβ1-42 for 24 hours, the neuronal cells were severely damaged, and the survival rate decreased to 18% (Figure 2, B and G). Similarly, the neuronal survival rate was not improved in neuron-microglia co-cultures treated with oAβ1-42 (Figure 2, C and G), which implies that unstimulated microglia have not protective effect against oAβ1-42 neurotoxicity. Administration of CpG to neuronal cultures or neuron microglia co-cultures induced no toxic change (Supplemental Figure S2A, see <http://ajp.amjpathol.org>). After 3 hours of treatment with 1 nmol/L, 10 nmol/L, or 100 nmol/L CpG, 5 μmol/L oAβ1-42 was added to neuron-microglia co-cultures for 24 hours. The neuroprotective effect was not evident in culture with 1 nmol/L CpG (Figure 2, D and G). However, microglia treated with 10 or 100 nmol/L CpG prevented neuronal cell death, and the neuronal survival rate was significantly improved reaching 53 and 62%, respectively (Figure 2, E–G). In neuronal cultures, CpG did not attenuate the neurotoxicity induced by oAβ1-42 (Figure 2G). Moreover, 100 nmol/L CpG attenuated oAβ1-42-induced neurotoxicity for 48 hours, whereas other TLR ligands such as peptidoglycan and lipopolysaccharide did not (Supplemental Figure S2B, see <http://ajp.amjpathol.org>). We conclude from these findings that CpG-activated microglia have neuroprotective effect against oAβ1-42 neurotoxicity *in vitro*.

### *CpG-Activated Microglia Increase the Clearance of oAβ1-42, Produce the Antioxidant Enzyme HO-1 and Aβ-Degrading Enzyme MMP-9, and Release Fewer Neurotoxic Molecules*

To elucidate the mechanisms of neuroprotection by microglia activated with CpG, we examined whether CpG increased the clearance of oAβ1-42. Western blot analysis revealed that there was no significant difference between the amount of oAβ1-42 present in the supernatants of neuronal cultures and in neuron-microglia co-cultures without CpG administration. (Figure 3A). However, CpG dose-dependently decreased the amount of oAβ1-42 in neuron-microglia co-cultures, especially treatment with 100 nmol/L CpG significantly decreased the amount of 3-, 4-, 8-, and 12-mer of oAβ1-42 (Figure 3, B and C). Moreover, we examined the effect of CpG on Aβ uptake by microglia alone at 1 and 24 hours time points. We found that CpG significantly enhanced microglial uptake of oAβ at both 1 and 24 hours (Supplemental Figure S3A, see <http://ajp.amjpathol.org>).

Because oxidative stress is a major component of oAβ1-42 neurotoxicity, we examined whether microglia activated with CpG express the antioxidant enzyme HO-1. CpG-activated microglia produced HO-1 in a dose-dependent manner. A total of 10 and 100 nmol/L CpG significantly increased the production of HO-1. The



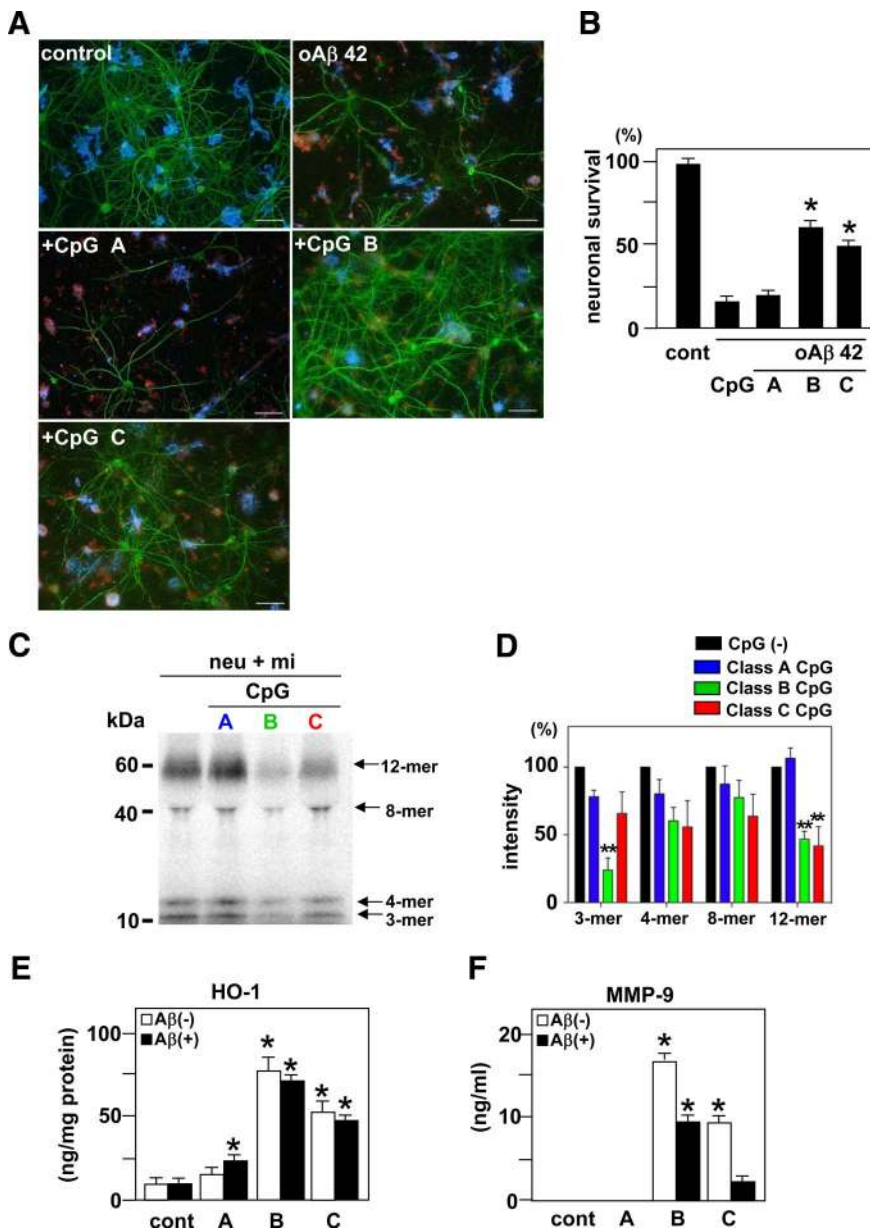
**Figure 3.** Clearance of oA $\beta$ 1-42 and the production of HO-1, MMP-9, and neurotoxic molecules by microglia activated with CpG. **A:** Western blot analysis of oA $\beta$ 1-42 in neuronal cultures (neu) and neuron-microglia co-cultures (neu + mi). Twenty-four hours after addition of 5  $\mu$ mol/L oA $\beta$ 1-42, oA $\beta$ 1-42 present in the supernatants of these cultures was detected by Western blotting. **B:** Western blot analysis of oA $\beta$ 1-42 in neuron-microglia co-cultures with CpG treatment. Neuron-microglia co-cultures (neu + mi) were treated with 5  $\mu$ mol/L oA $\beta$ 1-42 for 24 hours following 3 hours of treatment with 1, 10, or 100 nmol/L CpG. Microglia activated with CpG dose-dependently reduced the amount of oA $\beta$ 1-42 in the supernatants. **C:** Semiquantification of oA $\beta$ 1-42 in **B** by densitometric analysis. The amount of oA $\beta$ 1-42 in neuron-microglial co-cultures without CpG (black) was normalized to 100%. oA $\beta$ 1-42 in co-cultures treated with 1 nmol/L CpG (blue), 10 nmol/L CpG (green), or 100 nmol/L CpG (red) was calculated. \* $P$  < 0.05 and \*\* $P$  < 0.01 as compared with the intensity of oA $\beta$ 1-42 in neuron-microglia co-cultures without CpG. Each column indicates the mean  $\pm$  SEM ( $n$  = 6). The production of HO-1 (**D**) and MMP-9 (**E**) by microglia activated with CpG in the absence or presence of oA $\beta$ 1-42. After 3 hours of treatment with CpG, microglial cultures were treated with or without oA $\beta$ 1-42 for 24 hours. \* $P$  < 0.05 as compared with untreated controls. Each column indicates the mean  $\pm$  SEM ( $n$  = 3–5). **F:** The effect of HO-1 and MMP-9 on oA $\beta$ 1-42 neurotoxicity. Neuron-microglia co-cultures were treated with 100 nmol/L CpG in the presence of 10  $\mu$ mol/L tin-mesoporphyrin (SnMP) IX, a specific HO-1 inhibitor, or 50 nmol/L MMP-9 inhibitor for 3 hours, and then oA $\beta$ 1-42 was added to the cultures for 24 hours. Tin-mesoporphyrin IX, but not the MMP-9 inhibitor, decreased neuronal survival rate. \* $P$  < 0.05 as compared with untreated controls. Each column indicates the mean  $\pm$  SEM ( $n$  = 6–9). **G:** The suppressive effect of CpG on ROS production by oA $\beta$  in the neuron microglia co-cultures. After neuron-microglia co-cultures were treated with or without 100 nmol/L CpG for 3 hours, cells were loaded with fresh nerve culture medium containing 5  $\mu$ mol/L H<sub>2</sub>DCFDA-AM for 30 minutes. After washing, culture medium containing 5  $\mu$ mol/L oA $\beta$ 1-42 was added and the increment of the fluorescence was calculated at 5 minutes. \* $P$  < 0.05 as compared with untreated controls. \*\* $P$  < 0.05 as compared with co-culture cells treated with oA $\beta$ 1-42. Each column indicates the mean  $\pm$  SEM ( $n$  = 4). **H:** The measurement of TNF- $\alpha$  (**left**), nitrite (**middle**), and glutamate (**right**) produced by microglia activated with 100 nmol/L CpG with or without oA $\beta$ 1-42. After 3 hours treatment with CpG, microglial cultures were treated with or without oA $\beta$ 1-42 for 24 hours. \* $P$  < 0.05 as compared with untreated microglia. Each column indicates the mean  $\pm$  SEM ( $n$  = 7).

production levels were not influenced by exposure to oA $\beta$ 1-42 (Figure 3D). Since the anti-inflammatory cytokine IL-10 induces HO-1 expression by macrophages,<sup>29</sup> we also examined and confirmed that IL-10 induced HO-1 mRNA expression by microglia (Supplemental Figure S3B, see <http://ajp.amjpathol.org>). Although CpG induced IL-10 in microglia, the expression was suppressed by oA $\beta$ 1-42 treatment (Supplemental Figure S3C, see <http://ajp.amjpathol.org>).

MMP-9 is also thought to play a neuroprotective role in AD because it degrades both oA $\beta$  and fA $\beta$ . A total of 10 and 100 nmol/L CpG significantly induced MMP-9 pro-

duction in microglia with or without treatment of oA $\beta$ 1-42 (Figure 3E). To determine whether HO-1 and MMP-9 contribute to the neuroprotective effects of CpG-activated microglia, we applied the specific HO-1 inhibitor tin-mesoporphyrin IX (Frontier Scientific, Logan, UT) and MMP-9 inhibitor (Merck, Darmstadt, Germany). The neuroprotective effect of CpG was abolished by treatment with 10  $\mu$ mol/L tin-mesoporphyrin IX (Figure 3E). However, inhibition of MMP-9 with an MMP-9 inhibitor at 50 nmol/L did not influence the neuroprotective effect of CpG-activated microglia (Figure 3F). These results imply that HO-1 rather than MMP-9 may contribute to the





**Figure 4.** Protective effect of microglia activated with each subclass of CpG against oAβ1-42 neurotoxicity. **A:** Class B and class C, but not class A, CpGs exhibited neuroprotective effects against oAβ1-42 neurotoxicity in neuron-microglia co-cultures. After 3 hours incubation with 100 nmol/L class A, class B, or class C CpGs, oAβ1-42 was added to neuron-microglia co-cultures for 24 hours. Neurons were stained with anti-MAP-2 antibody (green). Aβ was stained with 4G8 (red), and microglia were stained with anti-CD11b antibody (blue). Scale bar, 50 μm. **B:** Neuronal survival rate was quantified. The viability of neurons in untreated co-cultures (control) was normalized to 100%. \**P* < 0.05 as compared with the co-cultures treated with oAβ1-42 alone. Each column indicates the mean ± SEM (*n* = 7). **C:** Western blot analysis of oAβ1-42 in neuron-microglia co-cultures with subclasses of CpG treatment. Neuron-microglia co-cultures (neu + mi) were treated with 5 μmol/L oAβ1-42 for 24 hours following 3 hours of treatment with 100 nmol/L class A, class B, or class C CpGs. Microglia activated with class B or class C CpGs reduced the amount of oAβ1-42 in the supernatants. **D:** Semiquantification of oAβ1-42 in **C** by densitometric analysis. The amount of oAβ1-42 in neuron-microglial co-cultures without CpG (black) was normalized to 100%. oAβ1-42 in co-cultures treated with class A CpG (blue), class B CpG (green), or class C CpG (red) was calculated. \*\**P* < 0.01 compared with the intensity of oAβ1-42 in neuron-microglia co-cultures without CpG. Each column indicates the mean ± SEM (*n* = 7). The production of HO-1 (**E**) and MMP-9 (**F**) by microglia activated with subclasses of CpG in the absence or presence of oAβ1-42. After 3 hours incubation with 100 nmol/L class A, class B, or class C CpGs, microglial cultures were treated with or without oAβ1-42 for 24 hours. \**P* < 0.05 as compared with untreated control cultures. Each column indicates the mean ± SEM (*n* = 3–5).

neuroprotection of CpG. Furthermore, treatment of oAβ significantly increased ROS production in the neuron microglia co-cultures. Pretreatment of 100 nmol/L CpG significantly suppressed ROS production by oAβ (Figure 3G).

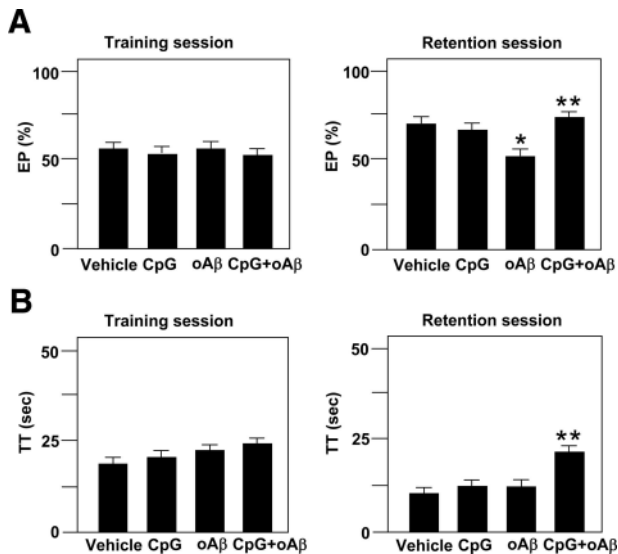
Although TLR4 ligand lipopolysaccharide generally increases microglial production of neurotoxic molecules including TNF-α, nitrite and glutamate; 100 nmol/L CpG did not induce these toxic molecules in microglia (Figure 3H).

#### *Microglia Activated with Class B and C, but not Class A CpG, Attenuate oAβ1-42 Neurotoxicity*

CpG ODNs are divided into three classes by their ability to induce IFN-α expression in plasmacytoid dendritic cells (class A) and to promote survival, activation, and maturation of B cells and plasmacytoid dendritic cell

(class B) or both (class C)<sup>30</sup>. We examined which class of CpG induces the neuroprotective effects of microglia. Class A CpG neither activated microglia nor induced neuroprotective effects against oAβ1-42 toxicity, whereas both class B and C CpGs activated microglia and significantly increased neuronal survival, to 58 and 49% following oAβ1-42 treatment, respectively (Figures 4, A and B). Western blot analysis revealed that class B CpG significantly decreased the amount of trimers and dodecamers of oAβ1-42 present in the supernatants of neuron-microglia co-cultures, and class C CpG significantly decreased dodecamers of oAβ1-42, whereas oAβ1-42 did not decrease by the administration of class A CpG (Figure 4, C and D). In addition, microglia activated with class B and C CpGs expressed HO-1 in both the absence or presence of oAβ1-42, whereas treatment with class A CpG only slightly increased HO-1 expression in the pres-





**Figure 5.** Effect of ICV injection of CpG on oA $\beta$ 1-42-induced cognitive impairment in the NORT. **A:** Exploratory preference (EP) and total exploring time (TT) (**B**) in the training session (**left**) and the retention session (**right**). NORT was performed 7 to 8 days after ICV injection of oA $\beta$ 1-42. Each column indicates the mean  $\pm$  SEM ( $n = 6-7$ ). \* $P < 0.05$  as compared with controls. \*\* $P < 0.05$  as compared with oA $\beta$ 1-42-injected mice.

ence of oA $\beta$ 1-42 (Figure 4E). Microglia activated with class B and C CpGs also produced MMP-9 (Figures 4F).

### ICV Injection of CpG Ameliorates oA $\beta$ -Induced Impairment of Recognition Memory in the NORT

To examine the role of CpG-activated microglia in cognitive dysfunction induced by oA $\beta$ 1-42, we investigated the effect of *in vivo* administration of CpG on the impairment of recognition memory in the NORT after ICV injection of oA $\beta$ 1-42. Mice injected with oA $\beta$ 1-42 displayed significantly reduced exploratory preference for the novel object in the retention session ( $F_{(3,21)} = 3.68, P < 0.05$ ; Figure 5A), although total exploration time in the training and retention sessions was unaffected. The result implies that oA $\beta$ 1-42 induces the impairment of recognition memory. Simultaneous injection of CpG with oA $\beta$ 1-42 significantly improved both exploratory preference ( $F_{(3,21)} = 3.68, P < 0.05$ ; Figure 5A) and total exploration time ( $F_{(3,21)} = 4.41, P < 0.05$ ; Figure 5B) in the retention session, although exploratory preference and total exploration time were unaffected in the training session.

### ICV Injection of CPG Ameliorates the Impairment of Associative Learning in the Cued and Contextual Fear-Conditioning Tests in Tg2576 Mice

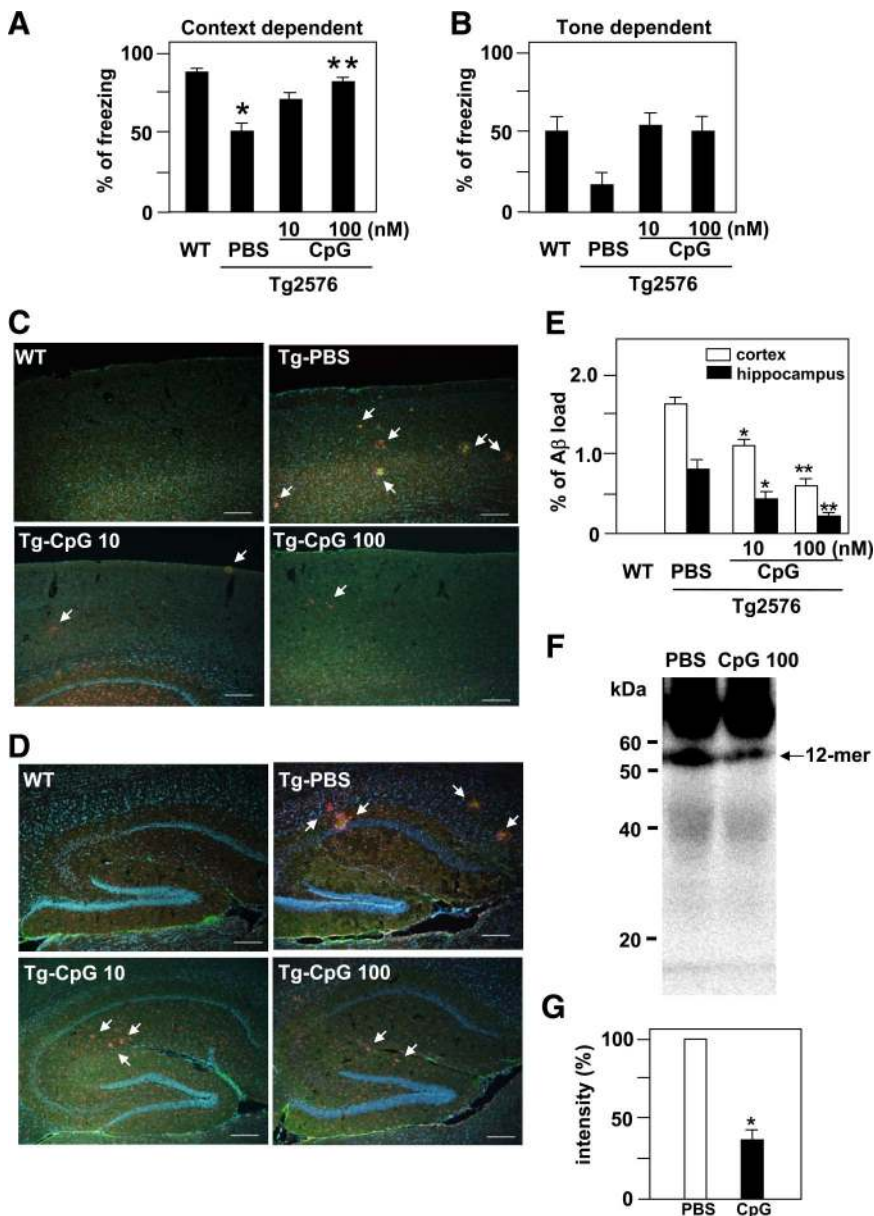
Next, we examined the effect of CpG on the cognitive function of Tg2576 mouse model of AD. We evaluated associative learning at the age of 10 months in a conditioned fear learning test. In the preconditioning phase (training), the mice hardly showed any freezing response. There were no differences in basal levels of freezing

response between the groups (data not shown). In the contextual learning test, wild-type mice showed a marked contextual freezing response 24 hours after fear conditioning (Figure 6A). However, vehicle-injected Tg2576 mice exhibited less of a freezing response in the contextual tests (Figure 6A), indicating an impairment of associative learning. The CpG (10 or 100 nmol/L)-injected Tg2576 mice were indistinguishable from wild-type mice, and the CpG treatment dose-dependently and significantly reversed the contextual freezing response as compared with vehicle-injected Tg2576 mice ( $F_{(3,35)} = 9.54, P < 0.05$ ; Figure 6A). In the cued (tone) learning test, although there was no significant difference in the cued freezing response at 24 hours after fear conditioning between wild-type and vehicle-injected Tg2576 mice, both injection of 10 and 100 nmol/L CpG showed a tendency to reverse the cued freezing response (Figure 6B). No alterations of nociceptive response were found in any of the mutant mice: there was no difference in the minimal current required to elicit flinching/running, jumping, or vocalization among the mice (data not shown). Then, we examined whether CpG decreased A $\beta$  deposits in the cortex (Figure 6C) and the hippocampus (Figure 6D) of Tg 2576 mice. No A $\beta$  deposits were seen in wild-type mice. Although A $\beta$  deposits (green) were abundant in the cortex and the hippocampus of vehicle-injected Tg2576 mice, ICV injection of CpG significantly decreased A $\beta$  deposits in both areas. Microglia (red) clustered around A $\beta$  deposits (Figure 6, C and D). CpG decreased A $\beta$  load in a significant, dose-dependent manner in both areas (Figure 6E). We examined oA $\beta$  in the soluble, extracellular-enriched fractions of the hemi-forebrains of mice and detected 12-mer oA $\beta$  in vehicle-injected Tg2576 mice by Western blotting. The 12-mer oA $\beta$  was strikingly and significantly decreased in 100 nmol/L CpG-injected Tg2576 mice (Figure 6, F and G).

### Discussion

Recent studies have proposed that oA $\beta$ 1-42 contributes to the neurotoxicity associated with AD. AD begins with subtle alterations of hippocampal synaptic efficacy before frank neuronal degeneration, and this synaptic dysfunction is caused by diffusible oA $\beta$ .<sup>31</sup> Disruption of hippocampal long-term potentiation and synaptic plasticity by oA $\beta$ 1-42 appears to involve Ca<sup>2+</sup> signaling,<sup>32</sup> oxidative stress mediated by an *N*-methyl-D-aspartate receptor,<sup>4,33</sup> and protein phosphatase 1.<sup>34</sup> In addition, oA $\beta$  interferes with insulin receptor function in hippocampal neurons and inhibits the activation of specific kinases required for long-term potentiation.<sup>35</sup> In the present study, we have confirmed that oA $\beta$ 1-42 exhibits more potent neurotoxicity than fA $\beta$ 1-42 in murine cortical cultures.<sup>5,36</sup> Therefore, decreasing or preventing formation of oA $\beta$ 1-42 is a potential therapeutic strategy against AD.

The precise role of microglia in oA $\beta$ 1-42 toxicity remains unclear. Microglia stimulated with A $\beta$  are reported to release proinflammatory cytokines via the nuclear factor  $\kappa$ B<sup>37,38</sup> and contribute to the pathogenesis of AD. However, in our experimental conditions, oA $\beta$ 1-42 neither



**Figure 6.** Effect of ICV injection of CpG on associative learning in the cued and contextual conditioning tests and A $\beta$  clearance in Tg2576 mice. The retention session was performed 24 hours after the training. Context-dependent (**A**) and tone-dependent (**B**) freezing times were measured at the age of 10 months. Each column indicates the mean  $\pm$  SEM (wild-type (WT) mice,  $n = 14$ ; vehicle-treated (PBS) Tg2576 mice,  $n = 9$ ; CpG (10 nmol/L)-treated Tg2576,  $n = 8$ ; CpG (100 nmol/L)-treated Tg2576,  $n = 8$ ). \* $P < 0.05$  compared with wild type. \*\* $P < 0.05$  as compared with vehicle-treated Tg2576 mice. **C:** A $\beta$  deposits in the cortex. A $\beta$  was stained with 4G8 (green), microglia stained with anti-CD11b antibody (red), and cell nucleus stained with Hoechst 33342 (blue). **White arrows** indicate A $\beta$  deposits surrounding by microglia. Scale bar, 200  $\mu$ m. **D:** A $\beta$  deposits in the hippocampus. Scale bar, 200  $\mu$ m. **E:** Quantification of A $\beta$  deposits in **C** and **D**. The ratio between the A $\beta$  deposits area and the total area of the analyzed region was multiplied by 100. \* $P < 0.05$  and \*\* $P < 0.01$  as compared with vehicle-treated Tg2576 mice. Each column indicates the mean  $\pm$  SEM ( $n = 3$ ). **F:** Western blot analysis of oA $\beta$  extracted from the hemi-forebrains of Tg 2576 mice. A 12-mer oA $\beta$  was detected in vehicle-treated Tg2576 mice and decreased in 100 nmol/L CpG-injected Tg2576 mice. **G:** Semi-quantification of 12-mer oA $\beta$  by densitometric analysis. The amount of 12-mer oA $\beta$ 1-42 in vehicle-treated Tg2576 mice was normalized to 100%. \* $P < 0.05$  as compared with vehicle-treated Tg2576 mice. Each column indicates the mean  $\pm$  SEM ( $n = 4$ ).

activated microglia nor induced the release of neurotoxic proinflammatory cytokines, NO, or glutamate. These results suggest that oA $\beta$ 1-42 is not acting to trigger of microglial neurotoxicity.

TLR signaling pathways contribute to phagocytosis of A $\beta$ . TLR2 acts as an endogenous receptor for the clearance of A $\beta$  by bone marrow-derived microglia.<sup>9</sup> Interestingly, a TLR4 mutation exacerbates A $\beta$  burden in mouse models of AD.<sup>39</sup> Thus, we investigated whether microglia activated with TLR ligands exert neuroprotective effects against oA $\beta$ 1-42 toxicity. Consequently, we found that TLR9 ligand CpG enhanced microglial neuroprotection. Furthermore, CpG exerted the neuroprotective effect of BV-2 microglial cell line against oA $\beta$  toxicity (data not shown). TLR9, which detects single-stranded DNA containing unmethylated CpG, is located in intracellular endosomal-lysosomal compartment. We confirmed that microglia expressed TLR9 at a higher level, whereas

astrocytes and neuronal cells expressed it at a lower level (data not shown). Thus, CpG mainly acts on microglia in the central nervous system. TLR7 and TLR8 are closely associated with TLR9. They are also located in endosomal-lysosomal compartment and detect single-stranded RNA. The ligands for TLR7 and TLR8 may also have some roles in microglial neuroprotection.

Western blot analysis revealed that microglia activated with CpG reduced the amount of oA $\beta$  present in the supernatant of treated cultures. Moreover, CpG was a potent inducer of antioxidant enzyme HO-1. The up-regulation of HO-1 in microglia by CpG treatment may lead to neuroprotection via suppression of ROS production by oA $\beta$ . HO-1, a member of the heat-shock protein family, is a microsomal enzyme that oxidatively cleaves heme to produce biliverdin, carbon monoxide, and iron.<sup>40</sup> A $\beta$  binds to heme to promote a functional heme deficiency, mitochondrial dysfunction, and neurotoxicity.<sup>41</sup> Amyloid

precursor protein also binds to HO, and oxidative neurotoxicity is markedly enhanced in cerebral cortical cultures from amyloid precursor protein Swedish mutant transgenic mice.<sup>42</sup>

HO-1 is reported to be induced by the anti-inflammatory cytokine IL-10.<sup>29</sup> Although CpG induced IL-10 in microglia in the absence of oA $\beta$ , IL-10 production was inhibited in the presence of oA $\beta$ . Therefore, HO-1 may be induced by a discrete mechanism independent from IL-10 in AD.

MMP-9, a protease that degrades A $\beta$ , is expressed at higher levels in the brains of AD patients and may play an important role in amyloid clearance by degrading both oA $\beta$  and fA $\beta$ .<sup>10</sup> MMP-9 expression is increased by serum amyloid A through formyl peptide receptor-like-1.<sup>43</sup> Although CpG stimulation induced MMP-9 in microglia, inhibiting MMP-9 pharmacologically did not affect neuroprotection by microglia. Thus, MMP-9 may not mainly contribute to the neuroprotection provided by CpG-activated microglia.

In the present study, CpG induced fewer neurotoxic molecules such as TNF- $\alpha$ , NO, and glutamate in microglia, whereas previous studies have reported that CpG-activated microglia produce TNF- $\alpha$ , IL-12, and NO<sup>44</sup> and induce neuronal damage.<sup>45</sup> The discrepancies between these studies and our experiments may be a consequence of differences in the concentrations of TLR ligands used. Higher concentration (10  $\mu$ mol/L) of CpG have been used for microglial activation in previous reports, whereas here we have used lower concentrations (1 to 100 nmol/L) of CpG.

In addition, we observed that the neuroprotective effect differs among CpG ODN classes. The responses of microglia to the different classes of CpG have not been fully understood. Here, we showed for the first time that class A CpG did not activate microglia, whereas class B and C CpGs induced neuroprotection by microglia that was mediated by clearance of oA $\beta$  and induction of HO-1. Three major classes of CpG ODN are structurally distinct. The structures of class A CpG include poly-G motifs at the 5' and/or 3' ends that are capable of forming very stable but complex higher-ordered structures and a central phosphodiester region containing one or more CpG motifs in a self-complementary palindrome. Class B CpG has a completely phosphorothioate backbone and does not form typically higher-ordered structures. Class C CpG has a phosphorothioate backbone, and 3' palindrome forms duplex.<sup>46</sup> These distinct structures of CpG ODN may reflect different microglial responses.

Finally, we examined the effect of CpG on oA $\beta$ 1-42 neurotoxicity in two different *in vivo* studies. The ICV administration of A $\beta$ 25-35 is reported to cause cognitive impairment in a NORT. Oxidative stress contributes to the onset of this cognitive dysfunction.<sup>23</sup> We found that injection of oA $\beta$ 1-42 also induced cognitive impairment as assessed by NORT. Surprisingly, one-time ICV injection of CpG improved both the cognitive impairment by oA $\beta$ 1-42 in NORT. The impairment of associative learning in Tg 2576 mouse model of AD was also effectively suppressed by ICV injection of CpG. We also confirmed that CpG treatment decreased A $\beta$  deposits and oA $\beta$  in

Tg 2576 mice. Our results concur with the recent study that a total of 14 i.p. injection of CpG into Tg 2576 mice beginning at the age of 6 weeks, and once a month, ameliorates AD-related pathology.<sup>28</sup> A $\beta$  plaques are reported to form extraordinarily quickly, over 24 hours. Within 1 to 2 days of a new plaque's appearance, microglia are activated and recruited to the site.<sup>47</sup> ICV injection of CpG may directly induce microglial activation via TLR9 and enhance microglial rapid uptake of oA $\beta$  through fluid-phase macropinocytosis as reported recently.<sup>48</sup> CpG may also enhance microglial phagocytosis of fA $\beta$  through formyl peptide receptor-like 2.<sup>11</sup> Such mechanisms of A $\beta$  clearance by CpG can decrease A $\beta$  plaque formation in Tg 2576 mice.

Recently, the therapeutic potential of CpG has generated great interest.<sup>46</sup> CpG offers a potent adjuvant activity that elicits a more effective immune response to infectious agents or tumors. A previous report<sup>49</sup> demonstrated that CpG strongly inhibits the effector phase of inflammatory arthritis. In addition, CpG can serve as a potent preconditioning stimulus and provide protection against ischemic brain injury.<sup>50</sup> Our findings suggest that CpG, especially class B and C, may also be effective therapeutic agents against oA $\beta$ 1-42 neurotoxicity in AD.

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